Estradiol increases IP\textsubscript{3} by a nongenomic mechanism in the smooth muscle cells from the rat oviduct

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Abstract

Estradiol (E\textsubscript{2}) accelerates egg transport by a nongenomic action, requiring activation of estrogen receptor (ER) and successive cAMP and IP\textsubscript{3} production in the rat oviduct. Furthermore, E\textsubscript{2} increases IP\textsubscript{3} production in primary cultures of oviductal smooth muscle cells. As smooth muscle cells are the mechanical effectors for the accelerated oocyte transport induced by E\textsubscript{2} in the oviduct, herein we determined the mechanism by which E\textsubscript{2} increases IP\textsubscript{3} in these cells. Inhibition of protein synthesis by Actinomycin D did not affect the E\textsubscript{2}-induced IP\textsubscript{3} increase, although this was blocked by the ER antagonist ICI182780 and the inhibitor of phospholipase C (PLC) ET-18-OCH\textsubscript{3}. Immunoelectron microscopy for ESR1 or ESR2 showed that these receptors were associated with the plasma membrane, indicating compatible localization with E\textsubscript{2} nongenomic actions in the smooth muscle cells. Furthermore, ESR1 but not ESR2 agonist mimicked the effect of E\textsubscript{2} on the IP\textsubscript{3} level. Finally, E\textsubscript{2} stimulated the activity of a protein associated with the contractile tone, calcium/calmodulin-dependent protein kinase II (CaMKII), in the smooth muscle cells. We conclude that E\textsubscript{2} increases IP\textsubscript{3} by a nongenomic action operated by ESR1 and that involves the activation of PLC in the smooth muscle cells of the rat oviduct. This E\textsubscript{2} effect is associated with CaMKII activation in the smooth muscle cells, suggesting that IP\textsubscript{3} and CaMKII are involved in the contractile activity necessary to accelerate oviductal egg transport.


Introduction

The canonical pathway by which estradiol (E\textsubscript{2}) affects its target cells comprises binding to estrogen receptors (ER) and modification of gene expression and protein synthesis (Nilsson et al. 2001). However, some E\textsubscript{2} effects cannot be blocked by inhibitors of transcription or translation, or are too rapid to be due to changes in gene expression. These features do not appear compatible with the classical genomic actions and are termed nongenomic (Lössel et al. 2003, Lössel & Wheling 2003). E\textsubscript{2} nongenomic actions often involve the activation of G protein-\textsubscript{a} inhibitory (G\textsubscript{a}i), stimulation of intracellular signal transduction pathways, including the generation of second messengers such as cAMP and IP\textsubscript{3}, and activation of protein kinase A (PKA) or phospholipase C (PLC) in the E\textsubscript{2}-target cells (Nadal et al. 2001, Wyckoff et al. 2001, Accconcia et al. 2005, Hill et al. 2010).

In the rat, a single injection of E\textsubscript{2} on day 1 of the cycle or pregnancy shortens oviductal transport of eggs from the normal 72–96 h to <24 h (Ort\textsuperscript{i}z et al. 1979). We have previously demonstrated that RNA and protein synthesis inhibitors did not block E\textsubscript{2}-induced oviductal egg transport acceleration in unmated rats indicating that E\textsubscript{2} accelerates oviductal egg transport by a nongenomic mechanism (Orihuela et al. 2001). This E\textsubscript{2} nongenomic pathway involves a previous conversion of E\textsubscript{2} to methoxyestradiols through the activation of catechol-O-methyltransferase (COMT) (Parada-Bustamante et al. 2007, 2010), ER and adenylyl cyclase (AC) (Orihuela et al. 2003), and sequential production of cAMP and IP\textsubscript{3} (Orihuela et al. 2003, 2006, 2013).

The rat oviduct is mainly composed of an intrinsic layer of smooth muscle fibre, and an innermost highly folded mucosa formed by epithelial and stromal cells, the endosalpinx (reviewed in Croxatto (2002)). Transport of oocytes along the oviduct depends on the interaction between the secretory activity of the epithelial cells and the contractile activity of the smooth muscle cells (Moore & Croxatto 1988a,b, Ríos et al. 2007). The regulation of muscular motility is influenced by E\textsubscript{2} and requires activity of adrenergic nerves (Helm et al. 1982), nitric oxide (Perez Martinez et al. 2000), endothelin (Parada-Bustamante et al. 2012), oxytocin (Jankovic et al. 2001) and prostaglandins (Wijayagunawardane et al. 2003). These factors activate intracellular...
signalling mainly associated with Ca\(^{2+}\), cAMP or IP\(_3\) (Jankovic et al. 2001, Barrera et al. 2004, Mohan et al. 2012). In this context, we have recently shown that, in the epithelial cells of the rat oviduct, E\(_2\) increased cAMP production between 3 and 6 h, although IP\(_3\) levels were not affected. Moreover, E\(_2\) increased cAMP in the oviductal epithelial cells by a nongenomic mechanism that requires coupling between ESR1 and G\(_\alpha\) and stimulation of AC (Oróstica et al. 2014). Previous research has also shown that E\(_2\) increased IP\(_3\) levels in primary cultures of smooth muscle cells from the rat oviduct (Oróstica et al. 2014).

As smooth muscle cells are the mechanical effectors for the accelerated oocyte transport induced by E\(_2\) in the oviduct (Croxatto 2002), this work determined the mechanism by which E\(_2\) increases IP\(_3\) in primary cultures of rat oviductal smooth muscle cells. Thus, we examined the effect of E\(_2\) on the IP\(_3\) levels in the smooth muscle cells under conditions in which protein synthesis, ER, G\(_\alpha\)i or PLC activity were blocked by selective inhibitors. The subcellular localization of ESR1 and ESR2 as well as the effect of selective agonists for ESR1 or ESR2 on the IP\(_3\) level was evaluated in the smooth muscle cells. Furthermore, expression of G\(_\alpha\)i in the oviductal smooth muscle cells was also determined. Finally, the effect of E\(_2\) on the activity of the enzyme associated to muscle contraction calcium–calmodulin protein kinase II (CaMKII) was determined in the primary cultures of smooth muscle cells.

Materials and methods

Animals

Locally bred Sprague-Dawley rats weighing 200–260 g were used. Animals were kept under controlled temperature (21–24 °C), and lights were on from 0700 to 2100 h. Water and pelleted rat chows were supplied ad libitum. Female mature rats were used in the estrous stage. The phases of the estrous cycle were determined by daily vaginal smears (Turner 1961) and all females were used after showing 2 consecutive 4-day cycles. The Ethical Committees of the Universidad de Santiago de Chile and the National Fund of Science (CONICYT-FONDECYT 1110662) approved the protocols for the care and manipulation of the animals.

Culture of primary smooth muscle cells from rat oviducts

For each replicate, 12 oviducts from six rats were excised and placed in pre-warmed Hank’s solution (Sigma Chemical) at pH 7.4. The whole oviduct was cut into small (4–8 mm\(^2\)) pieces in Hank’s solution and then the smooth muscle cells were mechanically removed from the rest of the tissue and treated with Collagenase, Type I (Invitrogen) for 1 h to further disaggregation of the cells. The cell suspension was centrifuged at 1200 g during 5 min, washed, and seeded into six-well tissue culture plates (Becton Dickinson, Franklin Lakes, NJ, USA) in DMEM/High Modified medium with 4.0 mM l-glutamine and 4.5 g/l glucose free of Phenol Red (Cat. No. SH30284.02, HyClone, Thermo Scientific, Waltham, MA, USA) supplemented with 10% (v/v) foetal bovine serum (Cat. No. SH30396.03, HyClone), 1 mM sodium pyruvate and 100 U/ml penicillin and 100 μg/ml streptomycin. Smooth muscle cells were incubated at 37 °C in an atmosphere of 5% (v/v) CO\(_2\) for at least 7 days to reach 75–80% confluence and their purity verified by immunofluorescence staining for cytokeratin (marker of epithelium cells), vimentin (marker of fibroblasts) or α-actin (marker of smooth muscle cells) antibodies.

Extraction and measurement of IP\(_3\)

Primary smooth muscle cell cultures were sonicated in 100 μl of ice-cold 1 M trichloroacetic acid (TCA) and an aliquot was taken to measure protein concentration by the Bradford assay using BSA dissolved in 1 M TCA as standard (Bio-Rad). The remaining homogenate was then centrifuged for 10 min at 1000 g at 4 °C. The pellet was discarded and the supernatant was incubated for 15 min at room temperature. TCA was removed from the supernatant with 0.5 ml of a solution 1,1,2-Trichloro-trifluoroethane (TCTFE, Sigma)-Trioctylamine (Sigma), 3:1 (v/v). Levels of IP\(_3\) were determined using IP\(_3\) \([^3H]\) radioreceptor assay Kit, Cat. No NEK064 (NEN Life Science Products, Boston, MA, USA). This kit is based on competition between-radioactive IP\(_3\) and a fixed quantity of \([^3H]\)IP\(_3\), for a limited number of calf cerebellum IP\(_3\) receptor binding sites. This allows the construction of a standard curve and the measurement of IP\(_3\) levels in unknown samples.

Western blot

Polyclonal antibodies that recognize the phosphorylated state of CaMKII on Thr286 (anti-phospho-CaMKII, Cell Signaling Technology, Beverly, MA, USA) or total CaMKII (anti-CaMKII,
Abcam, Cambridge, UK) were used to assess activation of CaMKII. Smooth muscle cells were processed by duplicate to determine the activity of the CaMKII protein. Cells were lysed in lysis buffer (20 mM Tris–HCl, pH 8.0, 137 mM NaCl, 1% Nonidet P-40 and 10% glycerol) supplemented with a protease inhibitor cocktail (Roche Diagnostics). The lysate was centrifuged at 4 °C for 10 min at 10 000 g and the pellet was discarded. Protein concentrations in the supernatant were measured by the Bradford assay (Bio-Rad). After boiling for 5 min, proteins (20 μg) were separated on 10% SDS–PAGE slab gels in a Mini PROTEAN electrophoretic chamber (Bio-Rad). Proteins resolved in the gels were electrophobted onto nitrocellulose membranes (Bio-Rad). The membranes were blocked 3 h in TTBS (100 mM Tris–HCl pH 7.5, 150 mM NaCl and 0.05% Tween-20) that contained 5% nonfat dry milk and were incubated overnight with 0.4 μg/ml rabbit anti-phospho-CaMKII (Cell Signaling Technology). The immunoreactive band of the immunoblot was visualized by incubation for 1 h with 0.04 μg/ml goat anti-rabbit IgG antibody (Chemicon International, Temecula, CA, USA) conjugated to HRP, followed by the Enhanced Western rabbit IgG antibody (Chemicon International, Temecula, CA, USA). Oviductal samples without anti-phospho-CaMKII or anti-CaMKII antibody were blocked 3 h in TTBS (100 mM Tris–HCl pH 7.5, 150 mM NaCl and 0.05% Tween-20) that contained 5% nonfat dry milk and were incubated overnight with 0.4 μg/ml rabbit anti-phospho-CaMKII (Cell Signaling Technology). The immunoreactive band of the immunoblot was visualized by incubation for 1 h with 0.04 μg/ml goat anti-rabbit IgG antibody (Chemicon International, Temecula, CA, USA) conjugated to HRP, followed by the Enhanced Western rabbit IgG antibody (Chemicon International, Temecula, CA, USA). Blots were stripped in 100 mM β-mercaptoethanol, 2% SDS and 62.5 mM Tris–HCl, pH 6.7 at 60 °C for 30 min, and reprobed with 0.2 μg/ml rabbit anti-CaMKII antibody and developed in a similar manner to ensure even loading. All blots were then digitalized and the relative level of phospho-CaMKII was normalized against total CaMKII. Oviductal samples without anti-phospho-CaMKII or anti-CaMKII antibody were included as negative controls.

Immmnonfluorescence

Smooth muscle cells were fixed in cold 4% paraformaldehyde in PBS pH 7.4–7.6 for 2 h, transferred to 10% (w/v) saccharose in PBS for 60 min at 4 °C and 30% (w/v) saccharose in PBS at 4 °C overnight. Then, they were blocked with 1% PBS–BSA for 120 min, and incubated with 0.8 μg/ml mouse anti-cytokeratin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), 2.5 μg/ml mouse anti-vimentin (Santa Cruz Biotechnology), 0.4 μg/ml mouse anti-α-actin (Santa Cruz Biotechnology) or 5.0 μg/ml mouse anti-Gα, (Santa Cruz Biotechnology) antibodies. After washing with PBS, the preparations were incubated for 2 h with 0.5 μg/ml Alexa fluor 568-conjugated goat anti-mouse IgG (Invitrogen). Sections were washed and counterstained with 1 μg/ml of Hoechst 33342 (Thermo Scientific, Rockford, IL, USA) washing again and then mounted in Fluoromount G. As negative controls, the primary antibody was replaced by preimmune serum. As positive control for Gα, we used samples of whole oviducts from rats on day 1 of the estrous cycle (Oróstica et al. 2013, 2014). All sections were visualized with an Optiphot Epifluorescence Microscope (Olympus).

Real-time PCR

Total RNA from primary secretory cell cultures was isolated using Trizol Reagent (Invitrogen). One microgram of total RNA of each sample was treated with Dnase I Amplification grade (Invitrogen). The single-strand cDNA was synthesized by reverse transcription using the Superscript III Reverse Transcriptase First Strand System for RT-PCR (Invitrogen), according to the manufacturer’s protocol. The Light Cycler instrument (Roche Diagnostics) was used to quantify the relative gene expression of the E2-target genes c-fos (Nilsson et al. 2001) in the oviductal smooth muscle cells; Gapdh was chosen as the housekeeping gene for load control. The SYBR Green I double-strand DNA binding dye (Roche Diagnostics) was the reagent of choice for these assays. Primers for c-fos were 5′-CCG AGATGG CCA ATC TAC TG 3′ (sense) and 5′-AGA AGG AAC CAG ACA GGT CC 3′ (antisense) and for Gapdh were 5′-ACC ACA GTC CAT GCC ATC AC 3′ (sense) and 5′-TCC ACC ACC CTG TTG CTG TA 3′ (anti sense). All real-time PCR assays were performed in duplicate. The thermal cycling conditions included an initial activation step at 95 °C for 5 min, followed by 40 cycles of denaturing and annealing-amplification (95 °C for 15 s, 59 °C for 30 s and 72 °C for 30 s) and finally one cycle of melting (60°C up to 95°C). The expression of c-fos was determined using the equation:

\[
\frac{2^{-\Delta\Delta CT}}{2^{-\Delta\Delta CT}}
\]
\[ Y = 2^{-\Delta CP} \] (16) where \( Y \) is the relative expression, \( C_p \) (crossing point) is the cycle in the amplification reaction in which fluorescence begins to be exponential above the background base line, \( -\Delta C_p \) is the result of subtracting \( C_p \) value of \( c\text{-los} \) from \( C_p \) value of \( gapdh \) for each sample. To simplify the presentation of the data, the relative expression values were multiplied by \( 10^3 \) (Livak and Schmittgen 2001).

**Statistical analysis**

Data for IP\(_3\) and CaMKII assays from cultured oviductal cells were replicated five times for each treatment (for each culture experiment, oviductal cells were recovered from a pool of six different rats). Statistical analysis was performed using a GraphPad Prism 5.0 Software program. All data are presented as mean \( \pm \) S.E.M. These data followed a non-normal distribution (Kolmogorov–Smirnov test) and significant differences between groups were determined through the use of variance analysis by Friedman’s test with subsequent post-hoc Wilcoxon signed-rank test. Significance was accepted at \( P < 0.05 \). On the other, the quantitative analysis of the ESR1 or ESR2 distribution was subjected to Kruskal-Wallis test, followed by Mann-Whitney’s \( U \) tests for pairwise comparisons when overall significance was detected. Significance was accepted at \( P < 0.05 \).

**Results**

**\( E_2 \) increased IP\(_3\) production through ER and PLC activation in the oviductal smooth muscle cells**

In each experiment, primary cultures of smooth muscle cells from rat oviducts were divided into the following treatment groups: i) ethanol + DMSO, ii) \( E_2 \) + DMSO, iii) ethanol + inhibitor and iv) \( E_2 \) + inhibitor. At 0, 1, 3, 6 or 9 h after treatment, cultured cells were processed to measure the concentration of IP\(_3\) as described in the ‘Materials and methods’ section.

Figure 3 shows that in the control groups, the IP\(_3\) level ranged from 151.6 \( \pm \) 26.3 to 193.5 \( \pm \) 37.3 fmol/\( \mu \)g of protein while in the \( E_2 \)-treated groups it ranged from 488.3 \( \pm \) 59.7 to 599.4 \( \pm \) 71.3 fmol/\( \mu \)g of protein. Administration of ICI 182780 or ET-18-OCH\(_3\) alone did not affect the basal IP\(_3\) production although blocked the \( E_2 \)-simulated IP\(_3\) increase at 6 h.

**ESR1 and ESR2 are localized in association with the plasma membrane of the oviductal smooth muscle cells**

Primary cultures of smooth muscle cells from rat oviducts with no treatment were processed by immunoelectron microscopy using specific antibodies for ESR1 and ESR2.

Figure 4A shows that immunoreactivity for ESR1 and ESR2 was found associated to the plasma membrane, cytoplasm and nucleus in the oviductal smooth muscle cells. Furthermore, Fig. 4B shows that the quantitative analysis of the ESR1 or ESR2 distribution was a higher number of ESR1 and ESR2-reacting gold particles in the nucleus than in the plasma membrane or cytoplasm of the smooth muscle cells.

**Activation of ESR1 but not ESR2 mimic the effect of \( E_2 \) on the IP\(_3\) production in the oviductal smooth muscle cells**

Primary cultures of smooth muscle cells from rat oviducts were treated with DMSO, PPT (\( 10^{-7} - 10^{-5} \) M) or DPN (\( 10^{-7} - 10^{-5} \) M) during 0, 1, 3, 6 or 9 h and processed to measure the concentration of IP\(_3\) as described in the ‘Materials and methods’ section. Other smooth muscle cells cultures were also treated with

![Figure 1](image-url) Expression of \( \alpha \)-actin in primary cultures of smooth muscle cells from rat oviducts. Representative photomicrographs of primary cultures of smooth muscle cells from rat oviducts were processed by immunofluorescence microscopy to detect expression of cytokeratin, vimentin or \( \alpha \)-actin. Note that and \( \alpha \)-actin (red) were only expressed in smooth muscle cells. Nuclei were stained with Hoechst 33342 (blue). Negative controls of the immunoreactivity were incubated with preimmune serum.
ActD and iv) E2

treatment groups: i) ethanol

G

DMSO or DPN 10^{-7} \text{ M and 6 h later processed by Real-Time PCR to determine the mRNA level of c-fos.}

Figure 5 shows that in the control group, the IP3 production ranged from 120.3 \pm 44.1 to 148.5 \pm 50.6 \text{ fmol/µg of protein} while treatment with PPT increased IP3 level in a dose-dependent manner at 6 h without any effect at 0, 1, 3 or 9 h. On the other hand, administration of DPN had no effect on the IP3 level at any time or concentration studied (Fig. 5), although it increased the mRNA level of c-fos (control: 51.4 \pm 11.3 vs DPN: 210.7 \pm 36.2 relative expression, n=5).

**Gα1 protein is not required for the IP3 production increase induced by E2 in the oviductal smooth muscle cells**

Primary cultures of smooth cells from rat oviducts were divided into the following treatment groups: i) ethanol + DMSO, ii) E2 + DMSO, iii) ethanol + PTX and iv) E2 + PTX. At 0, 1, 3, 6 or 9 h after treatment, cultured cells were processed to measure the concentration of IP3 as described in the ‘Materials and methods’ section. Note that ActD did not block the effect of E2 on the IP3 level. This experiment consisted of five replicates. *P<0.05 with respect to each treatment group.

**E2 induced activation of CaMKII in the oviductal smooth muscle cells**

Primary cultures from rat oviductal secretory cells were treated with ethanol or E2 \text{ 10^{-9} M and 6.5 h later the level of phosphorylated CaMKII (p-CaMKII) was assessed by immunoblot. As E2 increases the IP3 level at 6 h after order to assess the immunoreactivity of Gα1 in these cells. In addition, we used sections of whole oviducts from rats on day 1 of the oestrous cycle to corroborate presence of Gα1 in the oviductal tissues. This experiment was replicated five times.**

Figure 6B shows that immunoreactivity of Gα1 was not found either in the primary cultures of the oviductal smooth muscle cells or in the myosalpinx layer of the whole oviduct. However, Gα1 was expressed in the endosalpinx of the rat oviduct as previously reported by Orostica et al. (2014).

**Gα1 protein is not expressed in the smooth muscle cells from the rat oviduct**

Primary cultures from smooth muscle cells from rat oviducts were processed by immunofluorescence in

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treatment, we consider that 6.5 h is a reasonable time to evaluate activation of CaMKII downstream of the IP₃ increase.

Figure 7 shows that E₂ increased the level of p-CaMKII in comparison with the vehicle group.

Discussion

The contribution of the different cell phenotypes of the rat oviduct on the E₂ nongenomic pathway associated with the cAMP-IP₃ signalling and involved in the accelerated egg transport is recently being disclosed (reviewed in Orihuela et al. (2013)). Here we show that E₂ increased IP₃ levels in the oviductal smooth muscle cells by a nongenomic mechanism because suppression of mRNA and protein synthesis by ActD did not prevent the effect of E₂ on the IP₃ level. Moreover, the E₂ nongenomic pathway that increases IP₃ requires activation of ER and PLC since blockade of ER by ICI 182780 and PLC by ET-18-OCH₃ reverted the E₂-induced IP₃ increase in the oviductal smooth muscle cells. Previous works have shown that some E₂ nongenomic pathways are associated with changes in the turnover of inositol lipids that generates IP₃ from the hydrolysis of phosphatidylinositol 4,5-biphosphate in several cell systems (Kisielewska et al. 1996, 1997, Razandi et al. 1999, Ariazi et al. 2010). Our findings show for the first time that a nongenomic action of E₂ associated with PLC-IP₃ signalling is also present in the smooth muscle cells of the mammalian oviduct. The effect of E₂ on the IP₃ level occurred from 6 h and declined at 9 h, indicating a transient action on the PLC-IP₃ signalling in the smooth muscle cells. A rapid turnover of IP₃, inactivation of PLC or down-regulation of ER could explain the lack of effect at 9 h.

Figure 4 Subcellular localization of ESR1 and ESR2 in the primary cultures of smooth muscle cells from rat oviducts. (A) Representative photomicrographs of primary cultures of smooth muscle cells from rat oviducts processed by immunoelectron microscopy with gold labeled-antibodies for ESR1 or ESR2. Arrows show gold particle labeling localized to the plasma membrane (PM), cytoplasm (C) and nucleus (N). Negative controls of the immunoreactivity were incubated with preimmune serum. ICS = intercellular space. (B) Density of gold particles (X±SE) observed for ESR1 and ESR2 in the plasma membrane (PM), cytoplasm (C) and nucleus (N). Note that density of ESR1 and ESR2 in the PM were lower than in the C and N. a≠b≠c, P<0.05. Replicates of this experiment consisted of 12 cells from three different cell cultures (four individuals cells/well plate).

Figure 5 Effect of selective agonists for ESR1 (PPT) or ESR2 (DPN) on the IP₃ level in primary cultures of smooth muscle cells from rat oviducts. Primary cultures of smooth muscle cells from rat oviducts were treated with DMSO, 10⁻⁷, 10⁻⁶ and 10⁻⁵ M PPT or DPN during 0, 1, 3, 6 or 9 h and processed to measure the concentration of IP₃ as described in the ‘Materials and methods’ section. Note that PPT increased the IP₃ level in a concentration-dependent manner at 6 h after treatment while DPN had no any effect. This experiment consisted of five replicates. *P<0.05, **P<0.02 and ***P<0.01 with respect to each treatment group.
of effect E2 on the IP3 level at 9 h; however, this remains to be determined.

In contrast to other reports that showed a rapid increase of IP3 by E2 in rat vaginal epithelial cells and HEPG2 cells (Singh & Gupta 1997, Marino et al. 1998), we found that E2 has a time of latency of 6 h to exert its effects on the IP3 production in the smooth muscle cells from the rat oviduct. Differences in the expression of the ER isoforms or in the signalling pathways between the different cell phenotypes may explain the delayed response to E2 in the smooth muscle cells. We postulate that the E2 nongenomic action that increases IP3 appears as a secondary response to intracellular changes localized upstream of PLC activation in the oviductal smooth muscle cells. According to this assumption, we have recently shown that E2-induced IP3 increase is preceded by a cAMP decrease in smooth muscle cells of the rat oviduct (Oróstica et al. 2014). Alternatively, E2 may be first metabolized into 2-methoxyestradiol to increase IP3 production in the oviductal smooth muscle cells. In this context, various biological effects of E2 including regulation of egg transport in the rat oviduct or modulation of the antihypertensive and neuroprotective effects of E2, requires previous conversion from E2 to 2ME in its target organs (reviewed in Dubey & Jackson (2001) and Parada-Bustamante et al. (2015)).

Inhibition of the ER activity did not affect basal IP3 production in the oviductal smooth muscle cells, indicating that other ER-independent signalling pathways are acting to state basal IP3 level. In accordance with this idea, various signalling pathways such as Angiotensin-II, arachidonic acid, endothelin-1 and norepinephrine regulate production of IP3 (reviewed in Bolton (2006)). On the other hand, ET-18-OCH3 alone had no effect on the IP3 production in the oviductal smooth muscle cells. Since 13 mammal PLC subtypes have actually been reported (Rhee 2001), it is probable that basal IP3 production depends on an ET-18-OCH3-insensitive PLC. In this context, it has been found that ET-18-OCH3 is more effective at inhibiting membrane-associated PLC-β1 than PLC-γ1 localized in the cytosol of human fibroblasts (Powis et al. 1992).

Physiological effects of E2 are mainly influenced by the differential distribution of ESR1 and ESR2 in its target organs. Our results showing localization of ESR1 and ESR2 in the cell membrane, cytoplasm and nucleus

Figure 6 Effect of PTX on E2-induced IP3 increase and expression of Gαi in the oviductal smooth muscle cells. (A) Primary cultures of smooth muscle cells from rat oviducts were divided into the following treatment groups: i) ethanol + DMSO, ii) E2 + DMSO, iii) ethanol + PTX and iv) E2 + PTX. At 0, 1, 3, 6 or 9 h after treatment, cultured cells were processed to measure the concentration of IP3 as described in the ‘Materials and methods’ section. Note that PTX did not block the effect of E2 on the IP3 level. This experiment consisted of five replicates. *P<0.05 with respect to each treatment group. (B) Representative photomicrographs obtained from primary cultures of oviductal smooth muscle cells or whole oviduct processed by immunofluorescence to detect expression of Gai (red) is only expressed in the endosalpinx (mainly composed of epithelium cells) layer of the whole oviduct. Nuclei were stained with Hoechst 33342 (blue). Negative controls of the immunoreactivity were incubated with preimmune serum. L, lumen; E, endosalpinx; M, myosalpinx.

Figure 7 Effect of E2 on the CaMKII activation in primary cultures of smooth muscle cells from rat oviducts. Primary cultures of smooth muscle cells from rat oviducts were treated with ethanol or E2 and 6.5 h later CaMKII activation was determined by western blot and densitometry using anti-phospho-CaMKII or total CaMKII antibodies. Note that E2 treatment increased the relative phosphorylation of CaMKII in the smooth muscle cells. This experiment consisted of five replicates. *P<0.05 with respect to the control group.
GTPases participate in the activation of the PLC-pathway by which E2 increases IP3 in the oviductal smooth muscle cells. This is in keeping with previous works showing that the Gαi subclass is the probable that this enzyme could be part of the PLC-IP3 signalling cascades induced by estrogens to activate nongenomic actions in smooth muscle cells.

Several works have reported that E2 exerts its vasoprotective actions regulating the contractile tone of the vascular, airway and myometrium smooth muscle cells (Kisielewska et al. 1996, Townsend et al. 2010, 2012, Cairrão et al. 2012, Holm et al. 2013). These effects are mainly associated with changes in the intracellular Ca2+ mobilization associated with cAMP and IP3 signalling pathways (Kisielewska et al. 1996, 1997, Townsend et al. 2010, 2012). Since E2 increases the frequency of myosalpinx contractions that accelerate oviductal egg transport in the rat (Moore & Croxatto 1988a,b), we can speculate that this E2 effect involves IP3 production and activation of CaMKII in the smooth muscle cells. This E2 effect on the oviductal egg movement could be involved in the very early maternal–embryo interactions that occur in the uterine tissues necessary to the embryo implantation (Gomez & Muñoz 2015).

In summary, we have found that E2 increases IP3 by a nongenomic action operated by ESR1, and that involves activation of PLC in the smooth muscle cells of the rat oviduct. Furthermore, E2 activates CaMKII presumably downstream of the IP3 increase in the oviductal smooth muscle cells, suggesting that IP3 and CaMKII are probably that this enzyme could be part of the PLC-IP3 signalling cascades induced by estrogens to activate nongenomic actions in smooth muscle cells.

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In summary, we have found that E2 increases IP3 by a nongenomic action operated by ESR1, and that involves activation of PLC in the smooth muscle cells of the rat oviduct. Furthermore, E2 activates CaMKII presumably downstream of the IP3 increase in the oviductal smooth muscle cells, suggesting that IP3 and CaMKII are
involved in the contractile activity of the oviduct. These findings provide new evidence to understand the molecular mechanisms underlying the role of smooth muscle cells on the E2 nongenomic action that accelerates egg transport in the rat oviduct.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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